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Triple infection of *Wolbachia* in *Trichogramma ostriniae* (Hymenoptera: Trichogrammatidae)

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Abstracts: *Wolbachia* are common bacteria found in arthropods. *Trichogramma ostriniae* is the major *Trichogramma* species in maize fields in China and it has been reported that *Trichogramma* species harbored *Wolbachia*. In this study, the *Wolbachia wsp* and 16s rDNA gene sequences were used to detect the infection of *Wolbachia* in natural populations of *T. ostriniae*. The results indicated that *T. ostriniae* was triply infected with three strains of *Wolbachia* based on the *wsp* gene, *i. e.*, *wOstGDAa* (GenBank accession no. EU157103), *wOstGDAb* (GenBank accession no. EU157104) and *wOstGDB* (GenBank accession no. EU157105). Phylogenetic analyses showed that *wOstGDAa* and *wOstGDAb* belong to supergroup A, while *wOstGDB* belongs to supergroup B. An extensive survey of *Wolbachia* infection in natural populations of *T. ostriniae* revealed that nearly all individuals tested were infected with *wOstGDAa*, *wOstGDAb* and *wOstGDB*. This is the first report that nearly 100% of the individuals in the population were triply infected with *Wolbachia*. According to our results, we suppose that *Wolbachia* may transfer among different *Trichogramma* species when they share a host egg.

Key words: *Trichogramma ostriniae*; *Wolbachia*; triple infection; *wsp* gene

1 INTRODUCTION

Wolbachia pipientis are alpha proteobacteria that infect many species of arthropods. These bacteria are transmitted through the egg cytoplasm and alter reproduction in their arthropod hosts in various ways (O' Neill *et al.*, 1992; Werren *et al.*, 1995a). *Wolbachia* are widespread and common in insects. Polymerase chain reaction (PCR)-based surveys for *Wolbachia* have detected these bacteria in 16% - 76% of the insects (Werren *et al.*, 1995a; West *et al.*, 1998; Jeyaprakash and Hoy, 2000; Kittayapong *et al.*, 2000).

Multiple infection that the host individual harbors more than one strain of *Wolbachia* had been reported previously. Double infection by two different strains of *Wolbachia* had been found in various arthropod hosts (Jeyaprakash and Hoy, 2000; Werren and Windsor, 2000; Keller *et al.*, 2004; Narita *et al.*, 2007; Prakash and Puttaraju, 2007). Though not reported so much, triple infection and even infection of four or five strains of *Wolbachia* in one single host have also been reported recently (Jamnongluk *et al.*, 2002; Kondo *et al.*, 2002; Reuter and Keller, 2003; van Borm *et al.*, 2003).

The genus *Trichogramma* (Hymenoptera: Trichogrammatidae) are minute egg parasitoids. In *Trichogramma*, seventeen species are reported as infected with *Wolbachia* symbionts (de Almeida, 2004). But in all of these reported *Trichogramma* species there is no multiple infection phenomenon detected. Since the individuals of *Trichogramma* were very small and the density of *Wolbachia* infected may be low, it is not quite easy to extract the DNA of a single wasp and detect the infection rate of *Wolbachia* in *Trichogramma* species. Up to now, there have been only a few reports about the infection rates of *Wolbachia* in *Trichogramma* species. Goncalves *et al.* (2006) carried out a field survey of native *Trichogramma* species in the main processing tomato region of Portugal, and determined the prevalence of *Wolbachia* in these species. Five *Trichogramma* species were found and three of them were infected with *Wolbachia*. All the wasp broods belonging to *T. cordubensis* were infected, whereas low infection rates were found in *T. evanescens* (0.9% of the broods) and *T. turkestanica* (4.5% of the broods). There were no reports about the infection rates of *Wolbachia* in *Trichogramma* species in China. However, the distribution and infection rate of *Wolbachia* are very important for the research of *Wolbachia*. So in this study we investigated the distribution and infection rate of *Wolbachia* in the wild *T. ostriniae* populations collected in Guangdong

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2 MATERIALS AND METHODS

2.1 Field collection

The egg masses of corn borer, *T. ostriniae*, were collected from the maize field in Boluo county, the City of Huizhou, Guangdong Province (23° 10.639′N; 113°55.952′E) and provided by Institute of Plant Protection, Guangdong Academy of Agricultural Sciences. Each of the collected egg masses that were parasitized by *Trichogramma* was reared in the laboratory until the adult wasps emerged and then stored in 100% ethanol at −20℃ until *Wolbachia* presence tests.

The corn borer eggs that were not parasitized by *Trichogrammas* were also reared in the laboratory until the larvae hatched and were stored in 100% ethanol at −20℃ until *Wolbachia* presence tests.

2.2 Identification of *Trichogramma* species

Trichogramma wasps emerging from a single corn borer egg mass were generally assumed to belong to a single species. So from each brood a single male was used to identify the wasp at the species level using morphology (Lin, 1994). The *Trichogramma* individual is very small, so it is somewhat difficult to identify its species and sometimes the wasps emerged from the same host egg mass may belong to different species. So in order to ensure accurate results, the identification of each wasp used in this study was performed following a molecular method again. Species-specific primers TrichF/ To 545R were used for identification (Li, 2007).

2.3 DNA extraction

Total genomic DNA of each insect was extracted following the protocol described by Vavre *et al.* (1999b) with slight modifications. To avoid cross-contamination, we used disposable chips to extract DNA. The insect was washed in double distilled water and then homogenized in 200 μL extraction buffer [100 mmol/L Tris-HCl pH 7; 1.4 mol/L NaCl; 20 mmol/L EDTA; 2% hexadecyl-trimethylammonium bromide (CTAB)] and incubated at 65℃ for 1 h. Then added 0.1 μL RNase and incubated at 37℃ for 1 h. 500 μL chloroform-isoamylic alcohol (24∶1) was added before centrifugation for 15 min at 13 000 rpm. The supernatant was collected and gently mixed with double volume of 100% ethanol and tenth volume of Na-acetate (3 mol/L, pH 5.2). After precipitation over night at −20℃ and centrifuged for 20 min at 13 000 rpm, the precipitate of DNA collected was washed with 70% ethanol and air dried. Finally 20 μL 1×TE buffer was added to dissolve the DNA sample, which was then stored at −20℃ until test.

2.4 PCR amplifications

Three diagnostic PCRs were performed to amplify a fragment of the 28s rDNA gene of the insects and the 16s rDNA and *wsp* genes of *Wolbachia*.

The 28s rDNA gene is universally present in eukaryotes and highly conserved. The primers based on the 28s rDNA gene were used to check the quality of DNA extraction. The primers were forward (5′-TACCGTGAGGGAAAGTTGAAA-3′) and reverse (5′-AGACTCCTTGGTCCGTGTTT-3′). PCR cycling conditions were a 2 min pre-dwell at 94℃ followed by 38 cycles of 30 s at 94℃, 50 s at 58℃, 90 s at 72℃ and a post-dwell period of 10 min at 72℃. Samples negative for 28s rDNA gene were discarded. The positive samples were reamplified with 16s rDNA and *wsp* primers (81F/522R; 136F/691R) using the PCR conditions described by Zhou *et al.* (1998). The 16s rDNA primers, which forward (5′-CATACCTATTCGAAGGGATAG-3′) and reverse (5′-AGCTTCGAGTGAAACCAATTA-3′) were used to screen for *Wolbachia* infection. PCR cycling conditions were a 2 min pre-dwell at 94℃ followed by 38 cycles of 30 s at 94℃, 45 s at 55 ℃, 90 s at 72℃ and a post-dwell period of 10 min at 72℃.

PCRs were performed in 25 μL reaction volumes: 2.5 μL 10 × PCR buffer, 2.5 μL 25 mmol/L $MgCl_2$, 2 μL dNTPs (10 mmol/L each), 0.75 μL of 10 μmol/L of each primer and 1 uint *Taq* DNA polymerase. DNA extracts of *Wolbachia*-infected *Trichogramma* evanesceus were used as positive controls. Negative controls containing only double-distilled water were also included to check contamination.

2.5 Cloning and sequencing

PCR products of the 16s rDNA and *wsp* gene segments were purified using a DNA Fragment Purification Kit (Sangon). Purified PCR products were cloned in the plasmid vector pMD19-T (TaKaRa) and transformed into *Escherichia coli* DH5α-competent cells. The nucleotide sequences of selected clones were sequenced on an ABI automated sequencer (ABIP rism 377, USA). Both strands of plasmids were sequenced using universal primers (M13 +, M13 −) with forward and reverse reads. At least three independent clones were sequenced from each *Wolbachia* strain in order to identify polymerase errors.

2.6 Phylogenetic analysis

Sequences were analyzed using DNAMAN

v5.2.2. Then conduct blast searches in National Center for Biotechnology Information (NCBI) web to determine whether the sequences were *wsp* gene of *Wolbachia*. Three *wsp* sequences obtained in this study and 46 reference *wsp* sequences (Table 1) retrieved from GenBank were used to construct the phylogenic tree. The phylogenic analyses were performed using MEGA v4. 0. The tree was constructed using Neighbor-Joining and Maximum Parsimony models. Bootstrapping was performed with the heuristic option for 1 000 replications in the two models.

Table 1 *Wolbachia* group nomenclature and their GenBank accession numbers

Supergroup	Group	*Wolbachia* host species	Associated *Wolbachia* strains	Phenotype	GenBank accession no.
A	Mel	*D. melanogaster* (Aub)	wMel	CI	AF020063
		D. simulans (Coffs harbour)	wCof	NE	AF020067
		A. fuscipennis	wFus	T	AF071909
		D. melanogaster (Harwich)	wMelH	NE	AF020066
	AlbA	*A. albopictus*	wAlbA	CI	AF020058
	Mors	*G. morsitans*	wMors	?	AF020079
		N. vitripennis	wVitA	CI	AF020081
		G. centralis	wCen	?	AF020078
		C. peregrinus	wPer	T	AF071914
	Kue	*E. kuehnlella*	wKue	?	AF071911
		T. kaykai (LC110)	wKayA	T	AF071912
		T. ourarachae	wBou	Fec	AF071913
		T. ostriniae (BJ)	wOstA☆	?	AY633578
		T. ostriniae (GD)	wOstGDA☆	?	AY633581
		T. evanescens	wEvaB☆	?	AY390280
	Riv	*D. simulans* (Riverside)	wRi	CI	AF020070
	Uni	*M. uniraptor*	wUni	T	AF020071
	Ha	*D. sechella*	wHa	CI	AF020073
		C. cautella	wCauA	CI	AF020075
	Pap	*P. papatasi*	wPap	?	AF020082
	Aus	*G. austeni*	wAus	?	AF020077
	Dro	*T. drosophilae*	wDro	CI	AF071910
	Eva	*T. evanescens*	wEvaA☆	?	AY390279
B	Con	*T. confusum*	wCon	CI	AF020083
		L. striatellus	wStri	CI	AF020080
		T. bedeguaris	wBed	T/?	AF071915
	Dei	*T. deion* (TX)	wDei	T	AF020084
	Sib	*T. sibericum* (SIB)	wSib	T	AF071923
	Kay	*T. kaykai* (JT6-3)	wKayB	T	AF071924
		T. kaykai (LC110)	wKayLC	T	AF071927
		T. deion (SW436)	wDeiSW	T	AF071925
		T. nubilale	wNub	T	AF071926
	Div	*A. diversicornis*	wDiv	T	AF071916
	For	*E. formosa*	wFor	?	AF071918
	Ori	*T. orizicolus*	wOri	CI	AF020085

续表 1 Table 1 continued

Supergroup	Group	*Wolbachia* host species	Associated *Wolbachia* strains	Phenotype	GenBank accession no.
		D. rosae	wRos	T/?	AF071922
		Sfuscipes	wFu	T/?	AF071921
		C. cautella	wCauB	CI	AF020076
		E. staufferi	wSta	T/?	AF071919
		L. australis	wAus	T	AF071920
	Pip	*C. pipiens* (ESPRO)	wPip	CI	AF020061
		D. simulans (DSW/Mau)	wMa	?	AF020069
		A. albopictus (Houston)	wAlbB	CI	AF020059
		T. dendrolimi	wDen☆	?	AF394235
		T. chilonis	wChi☆	?	AY311486
	Vul	*A. vulgare*	wVul	F	AF071917

CI: Cytoplasmic incompatibility; F: Feminization; Fec: Fecundity increase; NE: No effect or rescue effect of CI; T: Thelytoky; T/?: *Wolbachia* and thelytoky are both observed, but no curing experiments have been performed; ?: Unknown; ☆:The Chinese strain.

3 RESULTS

3.1 Species identification

The products of the amplification of species-specific primers TrichF/To 545R were 545 bp in length. One male and one female wasps of each brood were identified (totally 101 individuals) and all of them were *T. ostriniae*.

3.2 Prevalence of *Wolbachia* in *Ostrinia furnalis*

The PCR results based on 16s rDNA and *wsp* genes indicated that there was no *Wolbachia* infected in the corn borer, *Ostrinia furnalis* collected in Guangdong Province.

3.3 Prevalence of *Wolbachia* in *T. ostriniae*

This targeted survey for *Wolbachia* infection in *T. ostriniae* using PCR amplification of the 16s rDNA gene revealed that there were two different 16s rDNA sequences in these insects. The phylogenetic analysis indicated that the two 16s rDNA sequences belonged to supergroup A and B, respectively (Fig. 1).

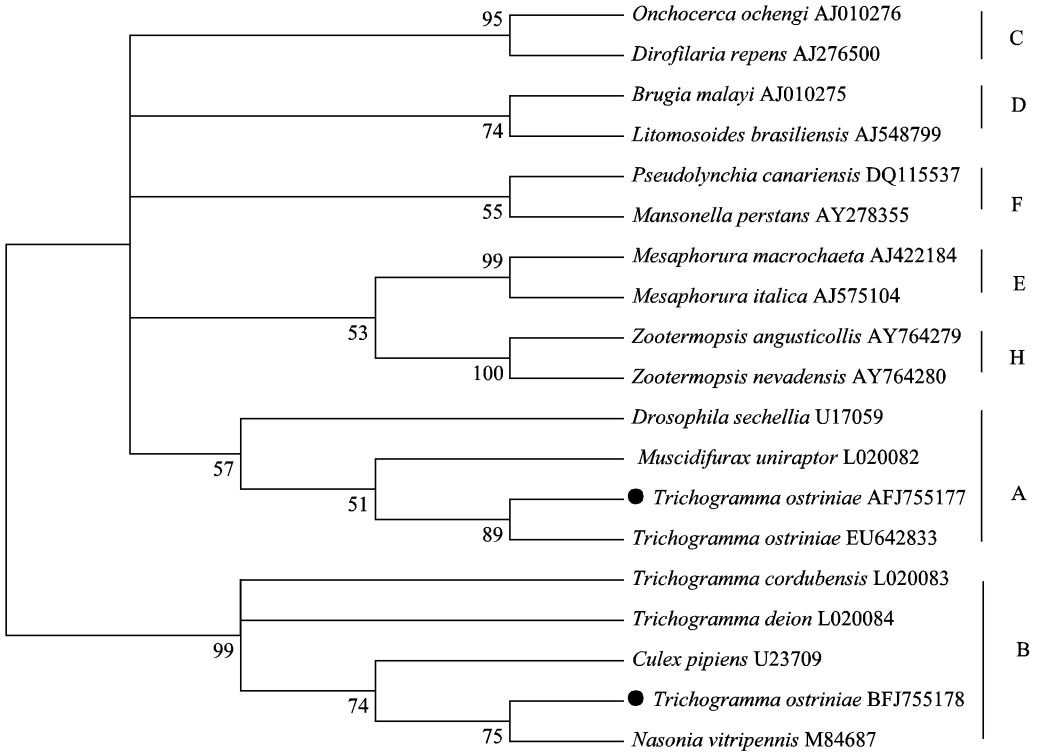

Fig. 1 Phylogenetic tree of *Wolbachia* based on 16s rDNA sequences constructed with NJ method in MEGA

The signed sequences were obtained in this study. The same below.

PCR amplification with *wsp* A-specific primers produced two distinct band (551 bp and 577 bp) from all the 101 field *T. ostriniae* individuals. PCR amplification with *wsp* B-specific primers produced one 442 bp bands from all the 101 field *T. ostriniae* individuals.

3.4 Phylogenetic Analysis

The identity between all the sequences we got and the *wsp* genes in GenBank was greater than 95%. So it is assured that all the sequences were the *wsp* genes of *Wolbachia*. Three distinct sequences were found to exist more than 2.5% differences from each other. The three *wsp* sequences were named as *wOstGDAa* (551 bp, GenBank accession no. EU157103), *wOstGDAb* (577 bp, GenBank accession no. EU157104) and *wOstGDB* (442 bp, GenBank accession no. EU157105). According to the taxon criterion of *Wolbachia* described by Zhou *et al.* (1998) that *Wolbachia* strains should belong to different *Wolbachia* groups when the differences between their *wsp* sequences were greater than 2.5%, the three *Wolbachia* strains belong to three different groups. All the tested *Trichogramma* individuals were found to harbor a triple infection with *Wolbachia*.

Phylogenetic analyses based on *wsp* sequences using different tree-building models yielded similar topology. Fig. 2 was the bootstrap consensus Neighbor-Joining tree. *Wolbachia* was divided into two supergroups, A and B. Both *wOstGDAa* and *wOstGDAb* belong to supergroup A. *wOstGDAa* belongs to group *Kue* and *wOstGDAb* belong to group *EvaA*. *wOstGDB* belongs to group *Pip* in supergroup B.

4 DISCUSSION

The *wsp* gene that encodes a surface protein of *Wolbachia* is evolving at a much faster rate than any other reported *Wolbachia* genes such as 16S rRNA gene and *ftsZ* genes (Zhou *et al.*, 1998). Based on the *wsp* gene, Zhou *et al.* (1998) classified the *Wolbachia* into two supergroups (A and B) and twelve groups (eight groups within supergroup A and four groups within supergroup B). The *wsp* gene is a very useful tool for identifying different *Wolbachia* strains. With the study of *Wolbachia* expanding, more and more *wsp* sequence information becomes available and the number of the groups is increasing.

Based on the PCR surveys of *wsp* gene, in this study all the 101 *T. ostriniae* individuals were infected with three groups of *Wolbachia*, *i. e.*, *wOstGDAa*, *wOstGDAb* and *wOstGDB*. The triple infection rate was 100% in both males and females. This indicates that the distribution of *Wolbachia* is equal between males and females. This paper is the first report that triple infection rate of *Wolbachia* reaches as high as 100% in both male and female *T. ostriniae* individuals in China maize fields. In our laboratory, we also investigated *Trichogramma* species collected from some other provinces and no triple infection was found in them.

Wolbachia are maternally inherited by vertical transmission through the host generations. However, sequencing of *Wolbachia* genes from different host species has revealed patterns of closely related *Wolbachia* strains distributed across widely divergent hosts (Rousset *et al.*, 1992; O'Neill *et al.*, 1992; Werren *et al.*, 1995b). The molecular phylogeny of *Wolbachia* was generally not parallel with their hosts. All of these strongly suggested that horizontal transmission of *Wolbachia* among different host species occurred (Werren *et al.*, 1995b; Schilthuizen and Stouthamer, 1998; Zhou *et al.*, 1998; Vavre *et al.*, 1999a). In recent years, some detailed experiments proved the horizontal transfer of *Wolbachia* (Heath *et al.*, 1999; Huigens *et al.*, 2000,2004; Shoemaker *et al.*, 2002). According to the analyses of the sequences we got in this study together with those cited from GenBank, we found that *wOstGDAa* and *wEvaB* infecting *T. evanescens* in China belong to the same group with the identity of 98.87%. *wOstGDAb* and *wEvaA* infecting *T. evanescens* in China belong to the same group with a high gene identity (98.75%). *wOstGDB* belongs to the same group with *wChi* and *wDen* which infect *T. chilonis* and *T. dendrolimi* in China respectively. Huigens *et al.* (2004) reported that horizontal transfer of *Wolbachia* occurred when infected and uninfected *Trichogramma* larvae shared a host egg. *T. ostriniae*, *T. evanescens*, *T. chilonis* and *T. dendrolimi* can synchronously parasitize so many hosts such as *Ostrinia furnalis*, *Clanis bilineata*, *Parnara guttata*, *Herse convolvuli*, *etc.* (Lin, 1994). So we can suppose that *Wolbachia* may transfer among these *Trichogramma* species when they share a host egg. But in this study, there was no *Wolbachia* detected in the corn borers. This indicated that the *Wolbachia* infected in *T. ostriniae* were not transferred from the host eggs.

In our opinions there are at least the following three questions that are valuable for further study:

Firstly, how does the triple infection of *Wolbachia* in *T. ostriniae* (GD) occur? Does it result from horizontal transfer of *Wolbachia* among the hosts of *T. ostriniae* and some other *Trichogramma* species?

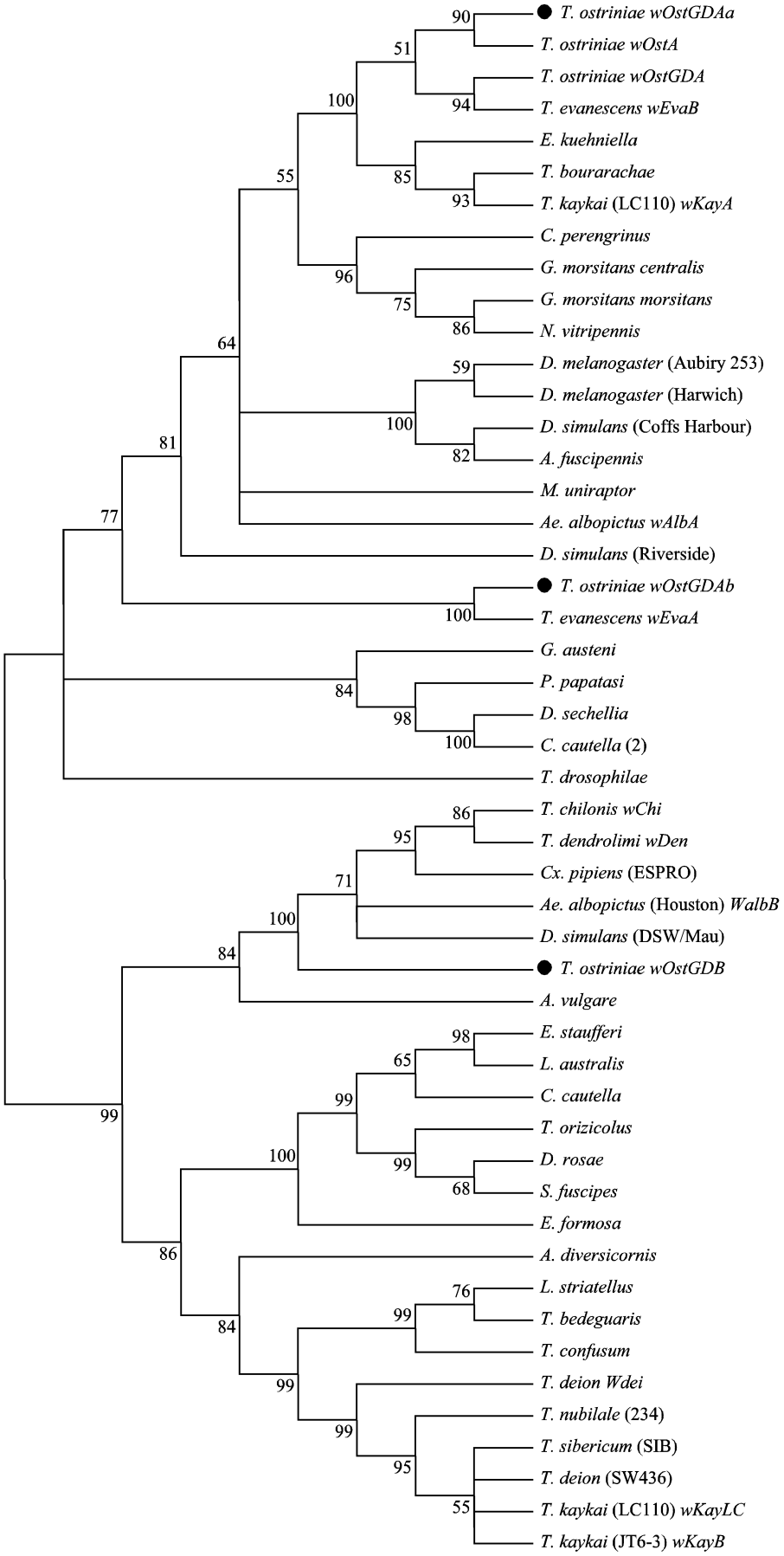

Fig. 2 Bootstrap consensus Neighbor-Joining tree of *wsp* gene

Secondly, *Wolbachia* infection is associated with a variety of productive anomalies in the host. It has been shown that *Wolbachia* infection causes cytoplasmic incompatibility in various insects, mites and isopods (Breeuwer, 1997; Hoffmann and Turelli, 1997), feminization of genetic males in isopods and moths (Bouchon *et al.*, 1998; Kageyama *et al.*, 1998), parthenogenesis in parasitoid wasps and a thrip (Stouthamer, 1997; Arakaki *et al.*, 2001) and male killing in beetles, butterflies and a fruit fly (Hurst *et al.*, 1999; Fialho and Stevens, 2000; Hurst *et al.*, 2000). It has been reported that *Wolbachia* infected in *Trichogramma* may induce thelytoky or increase the fecundity of the hosts (Stouthamer *et al.*, 1990; Girin and Boulétreau, 1995). However, in this study, the *T. ostriniae* were all sexual or arrhenotoky. What kind of impacts do the *Wolbachia* infected in *T. ostriniae* act on their hosts? We should do more research about these.

Finally, *wPip* and *wAlbB* which belong to the same group with *wOstGDB* were reported to induce CI in the hosts (Zhou *et al.*, 1998). Will the *wOstGDB* induce CI too? Is there any restrictive mechanism among the three *Wolbachia* strains? It is very important and valuable for us to study.

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Wolbachia 在玉米螟赤眼蜂内的三重感染

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摘要: *Wolbachia* 是一类广泛存在于节肢动物体内的共生菌。玉米螟赤眼蜂 *Trichogramma ostriniae* 是我国玉米田间的优势赤眼蜂种, 据报道, 赤眼蜂种内有 *Wolbachia* 感染。本文利用 *Wolbachia* 的 16s rDNA 和 *wsp* 基因引物通过 PCR 方法对玉米螟赤眼蜂的野生种群进行了调查, 发现以 *wsp* 基因为鉴定依据, 检测的所有个体都感染了 3 种 *Wolbachia* [*wOstGDAa* (GenBank accession no. EU157103), *wOstGDAb* (GenBank accession no. EU157104) 和 *wOstGDB* (GenBank accession no. EU157105)]。本文首次报道了野生赤眼蜂种群内 *Wolbachia* 的三重感染率几乎为 100%。根据本研究的结果, 可以推测当不同种赤眼蜂寄生同一寄主时, *Wolbachia* 可能会在不同赤眼蜂种间进行横向传播。

关键词: 玉米螟赤眼蜂; *Wolbachia*; *wsp* 基因; 三重感染; 横向传播

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